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(54)【発明の名称】　迅速分解性ＧＦＰ融合タンパク質および使用方法

(57)【要約】
グリーン蛍光タンパク質（ＧＦＰ）は遺伝子発現およびタンパク質局在化を測定する際のレポーターとして広く用いられている。本発明は、約１０時間以下の半減期、幾つかの態様においては４時間以下の半減期を有する融合タンパク質を提供する。そのようなタンパク質は、ＰＥＳＴ配列を含むマウスオルニチンデカルボキシラーゼ（ＭＯＤＣ）の分解性ドメインのＣ末端アミノ酸を、ＧＦＰの増強変異体（ＥＧＦＰ）のＣ末端に融合させることにより構築できる。トランスフェクションした細胞における融合タンパク質の蛍光強度はＥＧＦＰのものに類似するが、この融合タンパク質はＥＧＦＰと異なり、シクロヘキシミドの存在下で不安定である。ＭＯＤＣ領域の種々の変異により、異なる半減期をもつ、多様な目的に有用な変異体が得られた。

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
Background of invention [0002]
reference of related application this application -- the U.S. patent application 09th / No. 062 or 102 (April 17, 1998 application) -- it is continuation application a part
[0003]
Field of invention this invention relates to the field of the biochemical assay method and a reagent. this invention relates to the embellished fluorescence protein and those operation more at a detail.
[0004]
Explanation of the advanced technology Since the green fluorescence protein (green fluorescent protein, GFP) of the jellyfish (jellyfish) EKUORIA Victoria (Aequorea victoria) origin is green fluorescence which is easy to detect, in order to investigate a gene expression and protein localization, it is used widely. ; for which GFP fluorescence does not need a substrate or a cofactor, therefore this reporter can use it for many a kind and various cells. GFP is very stable protein, and since it may accumulate, it becomes often poisonous for a mammalian cell.
[0005]
Recently, a wild type GFP, and variation GFP It became clear that the three-dimensional structure of the crystallography-structure of S65T to GFP resembles a barrel (barrel) (Ormo et al.(1996) Science, 273:1392-1395;Yang, F., Moss, L.G.and Phillips, G.N.Jr.(1996) Nature Biotech, 14:1246-1251). This barrel consists of a beta sheet of dense (it is compact) reverse parallel structure. The alpha spiral containing a chromophore is shielded by the barrel by the center of a barrel. Variously [protease processing etc.], this dense structure makes GFP very stable under severe conditions, and makes GFP the general very useful reporter. On the other hand, the stability makes difficult measurement of a short-term event or a repetitive event.
[0006]
Much researches are done, in order to improve the property of GFP and to manufacture a useful GFP reagent for the purpose of [various] research. GFP of a new mold was developed by variation. In it, it is "hominization" GFP. DNA was contained and the protein product increased the composition in a mammalian cell (Cormack, et al.(1996) Gene, 173, 33-38;Haas, et al.(1996) Current Biology, 6,315-324; and Yang, et al.(1996) Nucleic Acids Research, 24, 4592 -4593 reference). One of such the hominization protein is "reinforcement (enhanced) green fluorescence protein" (EGFP). The version which emits the fluorescence of blue, cyanogen, and yellow was obtained by other GFP variation.
[0007]
The ornithine decarboxylase (ODC) is an enzyme important for the biosynthesis of polyamine. The rat ornithine decarboxylase is one of the shortest-lived protein in a mammalian cell, and has a half-life for about 30 minutes (Ghoda, et al.(1989) Science, 243, 1493-1495; and Ghoda, et al.(1992) Mol.Cell.Biol., 12, 2178-2185). It was judged with what the rat ornithine decarboxylase decomposes promptly being what is depended on unique composition of the C terminus. The part has a PEST array, i.e., the array advocated to be the feature of short-lived protein. It acts as the furan king (contiguity) of the PEST array including the field which is rich in a proline (P), glutamic acid (E), a serine (S), and a threonine (T) by the lysine, arginine, or histidine this [whose] is often a basic amino acid (Rogers, et al.(1989) Science, 234:364-68;Reichsteiner, M.(1990) Seminars in Cell Biology, 1:433-40).
[0008]
TbODC will become unstable if the C terminus of the rat ornithine decarboxylase is added to long-lived and quite stable; (Ghoda, et al.(1990) J.Biol.Chem., 265:11823-11826), however TbODC when the ornithine decarboxylase (TbODC) of Trypanosoma BURUSEI (Trypanosoma brucei) does not have a PEST array but it is discovered in a mammalian cell. Furthermore, if the deletion of the PEST content field of a C terminus is carried out from the rat ornithine decarboxylase, the prompt decomposition will be prevented (Ghoda, L., et al.(1989) Science, 243, 1493-1495).

[0009]
The advanced technology destabilized or runs short of short-lived GFP. this invention fills this request in the advanced technology.
[0010]
Outline of invention Quick metabolic-turnover nature (turnover) Or Destabilization GFP can be used for the research use which has not used GFP of the advanced technology. Using it as the gene reporter for analysis of Destabilization GFP of a transcriptional control and/or a cis-operation nature controlling element or a tool for studying proteolysis is included in such a use. Furthermore, development of the stable cell lineage which discovers a GFP gene becomes easy according to the quick metabolic turnover GFP. It is because poisonous GFP level is avoided since GFP protein is disassembled promptly.
[0011]
this invention offers fusion protein with the half-life shortened more remarkably than the thing of a wild type GFP. In one mode, the fusion protein which generated the destabilized protein is offered including EGFP united with the peptide. other modes -- setting -- the half-life of about 10 or less hours -- desirable -- the half-life of about 4 or less hours -- the half-life of about 2 or less hours and the fusion protein which has the half-life of about 1 or less hour still more preferably are offered more preferably The desirable mode of this invention of this viewpoint contains the PEST array content portion of the C terminus of EGFP and/or the rat ornithine decarboxylase (MODC). The concrete desirable mode of this invention includes EGFP-MODC 376-461, EGFP-MODC 376-456, EGFP-MODC 422-461, P426 A/P427A, P438A, E428 A/E430 A/E431A, E444A, S440A, S445A, T436A, D433A/D434A, and D448A.
[0012]
In the viewpoint of further others of this invention, the isolation DNA molecule which carries out the code of the fluorescence nature fusion protein with the half-life shortened more remarkably than the thing of a wild type GFP is offered. one mode of this invention of this viewpoint -- setting -- the half-life of about 10 or less hours -- the half-life of about 4 or less hours and the isolation DNA molecule which carries out the code of the half-life of about 2 or less hours and the fluorescence nature fusion protein which has the half-life of about 1 or less hour still more preferably more preferably are offered preferably In the desirable mode of this invention of this viewpoint, the isolation DNA molecule which carries out the code of the fluorescence nature fusion protein is a synthetic GFP gene containing the codon that in the high Homo sapiens protein of manifestation nature seen. [many] Furthermore, this invention offers the vector which may discover the isolation DNA molecule which carries out the code of the GFP fusion protein which the half-life shortened. In one mode of a vector, a vector contains an inductive promotor.
[0013]
In other modes of this invention, the method of carrying out the indicator of the cell by the transient GFP reporter is offered. In this method, a DNA vector including the isolation DNA which carries out the code of the GFP fusion protein which the inductive promotor and the half-life shortened is used. The transfection of this vector is carried out to a cell, a cell is cultivated under the conditions to which a promotor guides the transient manifestation of the GFP fusion protein of this invention, and, thereby, transient fluorescence level is obtained.
[0014]
Detailed description of invention this invention is destabilized and indicates the fluorescence protein which carries out metabolic turnover promptly in a cell and which was processed in genetic engineering. This fusion protein contains in a detail more the fluorescence protein which has the half-life which does not exceed about 10 hours, and the half-life which does not exceed about 2 hours most preferably. Preferably, fluorescence protein is chosen from the group which consists of EGFP, ECFP, and EYFP. In one mode, GFP processed in engineering is fusion protein of EGFP and the peptide which generates destabilization protein by the content. The example of such a peptide is the C terminus field of the rat ornithine decarboxylase (MODC). In the detailed example, the resolvability domain of the amino acid 422-461 of the rat ornithine decarboxylase was added to the C terminus of a reinforced type GFP variant (EGFP). Although the fluorescence intensity of the EGFP-MODC422-461 fusion protein in the cell which carried out the transfection was the same as that of the thing of EGFP, unlike EGFP, this fusion protein disappeared with time in the cell which carried out cycloheximide processing. The half-life of EGFP exceeded 24 hours to the half-life of the fluorescence of EGFP-MODC422-461 fusion protein having been about 2 hours. An ornithine-decarboxylase resolvability domain reduces the stability of EGFP remarkably.
[0015]
Quick metabolic-turnover type EGFP is equipped with at least four advantages in which EGFP is excelled. According to the prompt metabolic turnover of an EGFP-MODC fusion object, the toxicity over the cell which discovers this fusion protein decreases. Therefore, one advantage is being able to establish a stable cell lineage using DNA which carries out the code of the EGFP-rat ornithine decarboxylase. Furthermore, destabilized EGFP-

MODC decreases accumulation of EGFP. Analytic sensitivity may be blocked if fluorescence protein is accumulated. Therefore, the destabilized quick metabolic-turnover nature fusion protein gives the result of high sensitivity more. Furthermore, Destabilization EGFP can be used as a transient reporter for investigating an operation of a transcriptional control and/or a cis-operation nature controlling element. Finally, EGFP-MODC fusion protein can be used and the process accompanied by a multigene manifestation can be investigated.

[0016]

Furthermore, EGFP-MODC fusion protein has an advantage peculiar to use of EGFP. For example, especially use of EGFP in a medicine screening assay is advantageous. The fluorescence of GFP is because it can detect in a cell, without performing addition of the addition process which cost requires, for example, lysis, an exogenous substrate, or a cofactor, fixation of a cell manufacture object, etc. An example of such an assay screens a test compound about disturbance of a TNF activating pathway, i.e., the path which finally affects apotosis. Probably, the compound identified by this assay will be useful to control of the cell process which participates in cancer and inflammation.

[0017]

Furthermore, the reporter gene of this invention can be combined with various enhancer elements, and various biological processes, such as a response to a heat response, the response to heavy metal, glucocorticoid activation, or cAMP, can be supervised. Especially the destabilization EGFP has a useful gene to although [of the gene which controls unique biological phenomena, such as a circadian rhythm, in the generating process and the dynamic protein transportation which carry out a transient manifestation, localization of the protein in a cell, and a row / studying a cyclicity manifestation] periodic. other uses of the EGFP-MODC fusion protein in a screening assay -- actually -- this contractor -- obvious -- it will be .

[0018]

Furthermore, by an inductive promotor's use, the manifestation of EGFP-MODC fusion protein is made to activate or deactivate arbitrarily, and the construction object which discovers useful protein to cell-lineage research can be created. The GFP model of the advanced technology discovered GFP on the poisonous level which blocks the cytogenesis, therefore did cell-lineage research impossible. Furthermore, the fluorescence intensity of Destabilization EGFP can be used as a reporter for studying the reaction rate of the mRNA imprint from the promotor who had Destabilization EGFP adjusted at what time since it was the direct scale of gene-expression level.

[0019]

A term called "GFP" used in this specification expresses the fundamental green fluorescence protein of the EKUORIA Victoria origin, and in order to acquire fluorescence stronger against this, or different fluorescence of a color, advanced-technology type GFP processed in engineering is also contained. The array of EKUORIA Victoria GFP is Prasher, D.C., and et. It is indicated by al.(1992) Gene and 111:229-33.

[0020]

A term called "EGFP" used in this specification is Kain and et. al.(1995) Biotechniques and GFP "hominization was carried out" so that it might be reported to 19(4):650-55 are expressed. In order to optimize the codon "by which hominization was carried out" to the protein manifestation in a human cell, change made to the GFP nucleic-acid array is expressed.

[0021]

The term "the peptide which generates the destabilized protein" used in this specification expresses the array of the amino acid or peptide which promotes destabilization or the prompt metabolic turnover of the protein which is the part (namely, thing for which proteolysis is guided). The PEST array indicated on these specifications is one of such the arrays. Other known arrays are peptides which promote phosphorization and a protein-protein interaction in this technical field.

[0022]

A term called "EGFP-MODC" used in this specification expresses with the C terminus EGFP united with the rat ornithine-decarboxylase array.

[0023]

The EGFP-MODC fusion protein for which the term "P438A" used in this specification was exchanged by the alanine in the proline (proline in the PEST portion of this array) of the position 438 of a rat ornithine-decarboxylase array is expressed. The same nomenclature is used also about EGFP-MODC variant P426A/P427A;E428A/E430A/E431A;E444A;S440A;S445A;T436A;D433A/D434A; and D448A. Detailed explanation is shown in drawing 6 .

[0024]

The moiety of a fluorescence signal disappears from the fluorescence protein discovered in the cell, and the term

the "half-life" used in this specification expresses time for a moiety to remain. The term "Tc" used in this specification expresses a tetracycline. A term called "CHX" used in this specification expresses a cycloheximide.
[0025]
According to this invention, general molecular biology, microbiology, and the technology of recombinant DNA can be used within the limits of skill of the technology concerned. Such technology is fully explained into reference. For example, Maniatis and Fritsch & Sambrook and "Molecular Cloning:A Laboratory Manual"(1982);" DNA Cloning:A Practical Approach"Vol.I And II(D. volume on N.Glover, 1985);"Oligonucleotide Synthesis"(M. volume on J.Gait, 1984);"Nucleic Acids Hybridization" (B.) [D.Hames] & Volume on S.J.Higgins (1985); "Transcription and Translation"(B. volume on D.Hames & S.J.Higgins (1984));" Animal Cell Culture"(volume on R.I.Freshney (1986));" Immobilized Cells and Enzymes"(IRL press (1986));B.Perbal"A Practical Guide To Molecular Refer to Cloning" (1984).
[0026]
A "vector" is the replicon which reproduces by this the segment which was made to combine other DNA segments and was combined with it, for example, a plasmid, a phage, or a cosmid.
[0027]
A "DNA molecule" expresses the macromolecule form deoxyribonucleotide (an adenine, a guanine, a thymine, or cytosine) of a single strand form or a double strand helical. This term expresses only the molecule of primary and the secondary structure, and does not limit it to the Miyoshi form of either specification. Therefore, the double stranded DNA seen in a straight-line-like DNA molecule (for example, restriction fragment), a virus, a plasmid, and a chromosome is contained especially in this term.
[0028]
DNA" code array (coding array)" is a DNA array which is imprinted when it puts under control of a suitable regulatory sequence by in vivo one, and is translated into a polypeptide. The boundary of a code array is appointed by the translation stop codon of the start codon of an end, and 5'(AMINO)3' (carboxyl) end. Although cDNA of a prokaryotic-cell array and the eukaryotic-cell mRNA origin, the genomic DNA array of the eukaryotic-cell (for example, mammalian) DNA origin, and a synthetic DNA array are included in a code array, it is not limited to these. A polyadenylation signal and an imprint termination array may be located in 3' side of a code array. An imprint and a translational-control array are DNA regulatory sequences which a promotor, an enhancer, a polyadenylation signal, a terminator, etc. make discover a code array in a host cell, and/or adjust a manifestation.
[0029]
A "promotor array" is the DNA control region which RNA polymerase joins together in a cell and can start the imprint of a down-stream (the direction of 3') code array. It is the purpose which defines this invention, and a promotor array has a boundary attached by the three-dash terminal by the transcription initiation site, is extended for the upstream (the direction of 5'), and contains the base or element of the minimum number required to start an imprint on the level in which detection higher than the background is possible. A transcription initiation site and the protein joint domain which participates in combination of RNA polymerase are in a promotor array. Although it is not necessarily at a usual state, an eukaryotic-cell promotor includes a "TATA" box and the "CAT" box in many cases. Since the various vectors of this invention are driven, various promotors including the inductive promotor can be used.
[0030]
A term called the "restriction endonuclease" and the "restriction enzyme" which are used in this specification is a bacterial enzyme, and expresses what cuts double stranded DNA a specific nucleotide sequence or near the, respectively. When exogenism or different species DNA was introduced in a cell, the "transformation" or the "transfection" of the cell was carried out by such DNA. DNA for transformations is included in the genome of a cell, may be (carrying out covalent bond) and does not need to be incorporated. For example, in a prokaryotic cell, yeast, and a mammalian cell, DNA for transformations can be held on episome elements, such as a plasmid. As for the cell by which the transformation was carried out stably, DNA for transformations is included in a chromosome about an eukaryotic cell, therefore it is inherited to a daughter cell by chromosome replication. This stability is proved according to the capacity to establish the cell lineage or clone which consists of a daughter-cell group into whom an eukaryotic cell contains DNA for transformations. A "clone" is the cell population guided by mitotic division from the single cell or the common ancestor. A "cell lineage" is the clone of a primary cell which can be increased to stability over many generations by in vitro one.
[0031]
The "different-species" field of a DNA construction object is a DNA segment in which the identification in a bigger DNA molecule is possible, and does not accompany this big molecule in nature. For example, DNA which is not what is usually acting as the furan king of the mammalian genomic DNA within the genome of a source-of-supply

organism will act as the furan king of the gene, when a different-species field carries out the code of the mammalian gene. the construction whose gene portion from two different sources of supply (source) becomes together, and, as for different species DNA, generates a fusion protein product in other examples -- a code array in the living body is included With allele change or a natural variation event (event), the different-species DNA field defined as this specification is not generated.

[0032]

The term the "reporter gene" used in this specification is the code array combined with the different-species promotor or the enhancer element, and when the construction object is introduced into an organization or a cell, it expresses easily an assay and the array which can carry out a fixed quantity for the product. An imprint and a translational-control array are DNA regulatory sequences which make a code array discover in host cells, such as a promotor, an enhancer, a polyadenylation signal, and a terminator.

[0033]

As for the amino acid indicated on these specifications, it is desirable that it is "L" isomer type. However, "D" isomer type can be used instead of being L-amino acid residue, as long as the polypeptide holds the functional characteristic of the target immunoglobulin combination. NH2 expresses the isolation amino group in the amino terminus of a polypeptide. COOH expresses the isolation carboxy group in the carboxy end of a polypeptide.

[0034]

Therefore, the fusion protein obtained does not exceed about 10 hours, but this invention relates to the fusion protein containing GFP which has the short half-life of about less than 1 hour. In a desirable gestalt, GFP is EGFP. Preferably, fusion protein contains EGFP united with the PEST array content portion of the C terminus of the rat ornithine decarboxylase (MODC). MODC 376-461, MODC 376-456, MODC 422-461, P426 A/P427A, P438A, E428 A/E430 A/E431A, E444A, S440A, S445A, T436A, D433A/D434A, and D448A are contained in the example of representation of the PEST array content portion of the C terminus of the rat ornithine decarboxylase. An example of the GFP fusion protein of this invention has the array shown in array number:1.

[0035]

this invention relates to the isolation DNA molecule which carries out the code of the fusion protein containing the fluorescence protein chosen from the group which consists of EGFP, ECFP, and EYFP again. An example of the isolation DNA of this invention has the array shown in array number:2. this invention relates to the vector which may discover this isolation DNA molecule again. In one gestalt, a vector is a tetracycline regulation expression vector including an inductive promotor.

[0036]

this invention is the manufacture method of fluorescence protein, for example, the stable cell lineage which discovers GFP, and relates to a method including the process which carries out a transfection by the vector which indicates a cell on these specifications again. Furthermore, this invention is the method of carrying out the assay of activation or deactivation of a promotor, other imprints, or a translation element with transient fluorescence reporter protein. The transfection of the cell is preferably carried out by the expression vector containing the fusion protein which does not exceed about 10 hours and which has the half-life of less than 1 hour most preferably for less than 4 hours. Fusion protein is related with a method including the process which is under those promotors, an imprint, or the influence of a translation element, and detects;, existence of the fluorescence in those cells, un-existing, or an amount in that case. In this method, it is the scale of the fluorescence protein which the amount of the fluorescence which exists in a cell discovers. Quick and the direct method for measuring the effect of these imprints or a translation element detect the difference of the fluorescence intensity of the intercellular which discovers fluorescence protein under the various target imprints or a translation element. Furthermore, the cell which carried out the transfection can be processed with the purpose compound, and the additional process which measures the influence the purpose compound affects those imprints or a translation element can be carried out. Quick and the direct method for measuring the effect which the purpose compound exerts on the imprint or translation of fusion protein to discover detect change of the fluorescence at the time of processing a cell with the purpose compound.

[0037]

It is the way this invention furthermore investigates a cell lineage, and is related with a method including the process which carries out the transfection of the undifferentiated cell by the vector which can discover the fluorescence nature fusion protein which this invention destabilized, is made to increase these undifferentiated cells under the conditions from which an undifferentiated cell turns into a differentiated cell, and detects un-existing or existence of fluorescence in a differentiated cell. the cell localization element this invention is the method of using the fusion protein indicated on these specifications for a cell localization examination, furthermore, carry out the transfection of the cell by the expression vector containing the fluorescence nature fusion protein which does not exceed about 10 hours and which has the half-life of less than 4 hours preferably, and according [fusion protein] to

presumption in that case -- joining together -- **** --; -- a method including the process which make increase these cells and detects the position of the fluorescence in; and a cell offers
[0038]
Example 1 Construction of a DNA expression vector About cDNA which carries out the code of the C terminus of EGFP and Rat ODC (MODC), it is pfu. It was made to amplify by DNA polymerase (Stratagene, California La Jolla). 5'3 which includes HindIII array side' containing :SacII recognition sequence which made EGFP amplify by primer of couple side. In order to form the open reading frame containing the C terminus of Rat ODC, the deletion of the stop codon of EGFP was carried out from the C terminus. 5'3 which includes EcoRI array side' containing :HindIII recognition sequence which also made C terminus of Rat ODC amplify by primer of couple side. The rye gate of the two amplified PCR products was carried out by the HindIII part, and cloning of this fusion object was carried out into the pTRE expression vector and Tc regulation manifestation system (Gossen, M. and Bujard, H.(1992) Proc.Natl.Acad.Sci., 89:5547-5551).
[0039]
EGFP-MODC fusion protein was built using these methods. This EGFP-MODC376-461 fusion protein contained the perfect C terminus of the rat ornithine decarboxylase. Although EGFP-MODC 376-456 and EGFP-MODC 422-461 contained a part of rat ornithine-decarboxylase resolvability domain, both contained the PEST array. Furthermore, subsequently to an alanine, the key amino acid of the PEST array in fusion protein was mutated by the homology extending method (Rogers, et al.(1986) Science, 234:364-368). In a PEST variant, it is P426A/P427A ;P 438A;E428A/E430A/E431A;E444A;S440A;S445A;T436A;D433A/D434A; and D448A were contained.
[0040]
Example 2 Cell transfection The construction object DNA is refined and it is CHO because of measurement of proteolysis. The transfection was carried out into the K1-off cell. CHO A K1-off cell carries out the reserve transfection (pre-transfected) of the CHO cell to a tet-repressor in the fusion protein (tTA) of herpes-simplex-virus VP16 gene. The gene which carries out the code of the fusion protein on a pTRE vector (Gossen and Bujard, above-shown) can be made to discover by this reserve transfection, and an imprint is made to start by combining with the ornamentation CMV promotor in whom this subsequently contains a tet-repressor joint element. This combination can be intercepted by the tetracycline, therefore can control a manifestation by the tetracycline. DNA was introduced into these cells by clone FEKUCHIN (CLONfectin) (Clonetec Laboratories, Palo Alto). Functional analysis of the cell which carried out the transfection was carried out 24 hours after.
[0041]
Example 3 Fluorometric analysis The cell was cultivated on cover glass so that observation under a fluorescence microscope could be performed. After the transfection, in 37 degrees C, it incubated for 24 hours on cover glass, and, subsequently the cell was fixed for 30 minutes by the paraformaldehyde 4%. In order to carry out fluorescence inspection with Zeiss and AKISHI male Co-op (Zeiss Axioskop) model 50 fluorescence microscope, cover glass was put on slide glass. before paraformaldehyde fixation in order to measure proteinic metabolic turnover -- a cell -- time processings various by the cycloheximide with a last concentration of 100microg [/ml] -- it carried out
[0042]
For FACS analysis, the cell which carried out the transfection, and the cell which carried out cycloheximide processing were collected by EDTA treatment, and the cell pellet was re-suspended in 0.5ml PBS. Subsequently, fluorescence intensity analysis of the cell suspension was carried out by the FACS caliber (Calibur) (BEKUTON DIKKUSON, California San Jose). EGFP was excited by 488nm and luminescence was detected using 510 / 20 band pass filters.
[0043]
Example 4 Western blot analysis For the Western blot analysis, the contrast cell which carried out the transfection, and the cell which carried out cycloheximide processing were collected and ultrasonicated in PBS, and the lysis object was prepared. SDS-PAGE separated protein. After moving protein to a film, the monoclonal antibody to GFP detected the fusion protein of EGFP and MODC. Detection was visualized with the chemiluminescence detection kit (Clonetec).
[0044]
Example 5 Measurement of the stability of EGFP-MODC protein It is shown that the amino acid 376-461 of the C terminus of the rat ornithine decarboxylase guides decomposition of TbODC in a mammalian cell. In order to prove that this decomposition domain can also guide decomposition of EGFP, the rat ODC array was added to the C terminus of EGFP, and the 1st fusion EGFP-MODC construction object was created (drawing 1). This EGFP-MODC376-461 fusion construction object was made to discover by Tc regulation expression vector (pTRE). CHO After carrying out a transient manifestation in a K1-off cell, the fluorescence intensity of EGFP-MODC376-461 fusion protein was inspected under the fluorescence microscope. The fluorescence intensity of EGFP-MODC376-

461 fusion protein was very low (drawing 1). Although it was thought that the fluorescence of this protein depended a low on prompt decomposition comparatively, the EGFP fusion protein with such low signal intensity will not be useful to most research uses.
[0045]
If prompt decomposition actually participates in the lowness of the fluorescence of EGFP-MODC 376-461, the fluorescence intensity of fusion protein will be able to be raised by reducing the catabolic rate. The size of the C terminus extension array of the rat ornithine decarboxylase can determine catabolic rate. If the protein catabolic rate carried out [protein] low tolan KETO more is obtained by the deletion from the ends of a decomposition domain and the last 5 amino acid is removed from the rat ornithine decarboxylase by it, decomposition of the rat ornithine decarboxylase will fall remarkably (Ghoda, L., et al.(1992) Mol.Cell.Biol., 12, 2178-2185). When the TbODC fusion object was united with the small extended array rather than it began from amino acid 422, decomposition was slower than the long extended array rather than it began from amino acid 376 (Li, X. and Coffino, P.(1993) Mol.Cell.Biol., 13, 2377-2383). Therefore, two smaller extended arrays, i.e., one side, were added 422-461 to amino acid 376-456, another side was added to the C terminus of EGFP, and EGFP-MODC 376-456 and EGFP-MODC 422-461 were created (drawing 1). As for these fusion protein, both contain a PEST array. The fluorescence intensity of both fusion protein was measured by fluorescence microscope inspection after the transfection. It was shown that a result has relative fluorescence intensity with both higher (especially fusion protein EGFP-MODC 422-461) than EGFP-MODC 376-461. The fluorescence of the fusion protein of this latter is similar to the thing of EGFP so that drawing 1 may show.
[0046]
Example 6 Detailed elucidation of the stability of EGFP-MODC422-461 protein Subsequently it investigated whether the C terminus extension array of amino acid 422-461 could guide EGFP decomposition by in vivo one. For this reason, it is CHO first about a construction object. The half-life of fusion protein was measured by carrying out a transient transfection into a K1-off cell, and intercepting protein synthesis by the cycloheximide (CTX). 24 hours after a transfection and a cell -- a 100microg [/ml] cycloheximide -- 0, 1, 2, and 3 -- and it processed for 4 hours Fluorescence microscope inspection investigates change of the fluorescence intensity of a cell which carried out the transfection, and a result is shown in drawing 2 . The fluorescence intensity of the fusion protein in a cell fell gradually in connection with cycloheximide processing becoming long. This shows that EGFP-MODC422-461 fusion protein is unstable. After processing by the cycloheximide for 4 hours, the fluorescence intensity which exists as compared with the fluorescence intensity of the cell of time zero was under a half. These results show that the fluorescence intensity of this fusion protein is less than 4 hours.
[0047]
Subsequently, EGFP-MODC 422-461 was compared with EGFP in the same assay. There was no change significant 4 hours after a protein synthesis halt in the EGFP fluorescence intensity of the cell which carried out the EGFP transfection (drawing 2). As for this, the half-life of EGFP shows a ******** from 4 hours. EGFP is stable protein and this [the protein product's of an EGFP-MODC422-461 construction object] corresponds with other reports of GFP which support the conclusion of being unstable, when discovered in a mammalian cell.
[0048]
In order to measure more precisely EGFP-MODC fusion protein and the half-life of EGFP, the fixed quantity of the change of the fluorescence of these 2 protein was carried out by the flow cytometry. By the cycloheximide, the cell which carried out the transfection was collected by EDTA, 0, 1, 2, and after processing for 3 hours, and 10,000 cells were used for FACS analysis. The fluorescence of;, i.e., fusion protein, which corresponded with fluorescence microscope observation decreased gradually in connection with cycloheximide processing becoming long (drawing 3).
[0049]
It is shown that the graph of drawing 4 gathered FACS data and maintained fluorescence 2 hours after the cycloheximide processing of about 50% of an unsettled cell. This shows that the half-life of this fusion protein is about 2 hours. Analyzing similarly the cell which carried out the EGFP transfection, the result showed that the fluorescence of EGFP did not change intentionally on the occasion of cycloheximide processing. In addition, the EGFP cell was actually maintaining many fluorescence from 80% after cycloheximide processing of 4 hours as compared with the unsettled EGFP cell. That is, the half-life of an EGFP fusion object is shortened appropriately, and is intentionally longer than 4 hours. [of the half-life of EGFP]
[0050]
Cycloheximide processing is poisonous for a cell, and since apotosis is started, it is inextensible from 4 hours. However, since the inductive manifestation system used for these examinations is adjusted by the tetracycline (Gossen, M. and Bujard, H.(1992) Proc.Natl.Acad..Sci., 89:5547-5551), EGFP composition can stop only by adding

a tetracycline. In order to measure the half-life of EGFP more precisely, the fluorescence intensity of the cell which carried out the EGFP transfection was supervised by both under existence of a tetracycline and un-existing 24 hours after. EGFP was made to discover first for after [a transfection] 24 hours. Subsequently, the cell which carried out the transfection was cultivated under existence of a tetracycline or un-existing for further 24 hours, and it collected to analysis by the flow cytometry. The difference of fluorescence intensity was not detected between the cells (-TC and +TC, bottom panel of drawing 3) of these 2 type. This shows that fluorescence did not change in 24 hours after EGFP protein synthesis interception. In these results, the half-life of EGFP shows a ******** from 24 hours.

[0051]

In order to investigate whether the half-life of EGFP and the EGFP fusion protein of this invention correlates with the amount of fluorescence, disassembly of fusion protein was supervised by the Western blot analysis. Both the EGFP transfection cell used for the flow cytometry of drawing 3 and the EGFP-MODC422-461 transfection cell were used also for the Western blot analysis by the monoclonal antibody to GFP. As shown in drawing 5 , a change detectable on the EGFP protein level of the intercellular processed by the cycloheximide for 0 to 3 hours was not seen. It is shown that this has stable EGFP during cycloheximide processing of 3 hours.

[0052]

The GFP monoclonal antibody detected similarly the EGFP fusion protein of this invention which carried out MODC ornamentation. The sizes of fusion protein were about 31 kDa(s). However, unlike EGFP, EGFP fusion protein was unstable. Being under the half of contrast, and the half-life of this fusion protein being less than 1 hour, and dealing in; fact that the level of fusion protein fell remarkably by the time of the cycloheximide processing end of 3 hours, and the EGFP fusion protein, with which it remains in 1 hour is shown. The difference of the half-life measured using a flow cytometry and the Western blot analysis is based on the fact that both Premature (namely, non-fluorescence nature) GFP and the maturation GFP are detected, by Western analysis. However, formation of an EGFP chromophore is after an imprint and advances in the half-activity term for about 25 minutes (half time) (Cormack, et al.(1996) Gene, 173, 33-38). The GFP content is [in / the fluorescence level / about this invention] important as not protein level but a reporter. -- The half-life of EGFP fluorescence is more important. The fluorescence half-life of in vivo EGFP-MODC 422-461 is about 2 hours.

[0053]

: whose amino acid sequence of EGFP-MODC422-461 protein is as follows -- [Formula 1]

[0054]

: whose DNA array which carries out the code of the EGFP-MODC422-461 protein is as follows -- [Formula 2]

[0055]
Example 7 Analysis of a PEST array The C terminus of the mouse ornithine decarboxylase includes the PEST array of amino acid 423-449. There are three proline residues, four glutamic-acid residues, two serine residues, and one threonine residue (drawing 6). In order to evaluate the intervention of each amino acid in the PEST motif to proteolysis speed, each Pro, Glu and Ser, and Thr residue of a PEST field of EGFP-MODC fusion protein were mutated to Ala. Decomposition was supervised by change of fluorescence. It is CHO about each construction object. The transient transfection was carried out into the K1-off cell. Under existence of a cycloheximide, 0, 2, and after processing for 4 hours, the cell was collected by cytophotometry analysis. Data are shown in drawing 7 .
[0056]
The variation of the proline residue of amino acid 438 stabilized protein. In addition, the rate of a fluorescence sexual cell was higher than 60% after cycloheximide processing of 4 hours. Therefore, there are more half-lives of a P438A variant than the double precision of the half-life of EGFP-MODC 422-461. This shows that it contributes to destabilization of fusion protein, when a proline exists in 438. The variation of the proline of the amino acid positions 426 and 427 did not extend a half-life. The half-life of P426 A/P427A is actually still shorter than the thing of EGFP-MODC 422-461. This shows that these proline residues may stabilize protein. the same result -- fusion construction -- it was obtained by the variation to a glutamine and a serine in the living body The cell which; with the half-life of variant E444A and S440A longer than the thing of EGFP-MODC 422-461 however E428A/E430A/E431A, and S445A became instability more, and was maintaining fluorescence after processing of 2 hours was only 20% or 29%.
[0057]
It often acts as the furan king of the PEST array by the basic amino acid (Rogers, et al.(1989) Science, 234:364-368). In order to show that these furan king amino acid residues are participating in proteinic instability, the histidine 423, the arginine 449, and the histidine 450 were mutated to the alanine. When the arginine and histidine of 449 and 450 were replaced by the alanine, proteinic stability increased remarkably. This suggests that these 2 amino acid is required for efficient proteolysis. Even if it mutated His of amino acid 423, proteinic stability did not change.
[0058]
Example 8 Cell lineage which discovers dEGFP by the Tet manifestation system CHO K1-tet The transfection of

the off cell was carried out by pTRE-EGFP-MODC 422-461 and pTK-hygromycin. The transfection was performed by the clone FEKUCHIN (CLONfectin) kit (Clonetec). The cell which carried out the transfection was chosen under existence of 200microg [/ml] hygromycin, and the resistance colony was screened per fluorescence under the fluorescence microscope. The single colony of a fluorescence sexual cell was moved to the respectively new plate. The destabilization EGFP in the cell by which the transfection was carried out stably was able to be adjusted by the tetracycline like the case of the cell which carried out the transient transfection, and decomposition was able to be detected by adding a cycloheximide and intercepting protein synthesis. The obtained cell lineage by which the transfection was carried out stably can be used for analysis of a large number including medicine screening. For example, the medicine which intercepts imprint guidance of Destabilization EGFP during the shift to tetracycline un-existing from tetracycline existence, or the medicine which checks proteolysis after cycloheximide addition can be screened using it.

[0059]

Example 9 Destabilization ECFP and EYFP CFP (cyanogen) and YFP (yellow) are the variants of the color of GFP. 6 and four kinds of variants are contained in CFP and YFP, respectively. In CFP, they are Ser65Gly, Val168Leu, Ser72Ala, and Thr203Tyr at Tyr66Try, Phe66Leu, Ser65Thr, Asn145Ile, Met153Thr and Val163Ala, and a row. The code of CFP (ECFP) and YFP (EYFP) which were reinforced is carried out by the gene containing a Homo sapiens optimization codon. ECFP is excited by 433nm and emits light by 475nm. EYFP is excited by 523 or 488nm, and emits light by 527nm.

[0060]

By the same method as the above for creating dEGFP, dECFP and dEYFP were created by adding the C terminus of the mouse ornithine decarboxylase to the C terminus of each of these 2 protein (drawing 8). The transfection of pTRE-dECFP and pTRE-dEYFP was carried out into the CHO/tTA cell, protein synthesis was suspended by addition of CHX, and the half-life of dECFP and dEYFP was measured by carrying out fluorescence microscope inspection. Both protein was unstable under existence of CHX. The half of the fluorescence of these 2 protein disappeared 2 hours after processing by CHX. This shows that the half-life of these protein was substantially shortened as compared with the wild type.

[0061]

The color variant of these two kinds of destabilization protein can be used together because of 2 color detection. For example, a dECFP gene can be combined with a NFkB junction sequence, and a dEYFP gene can be combined with a NFAT junction sequence. If these two reporter fusion objects are introduced into a single cell, two imprint factors or two signaling paths are simultaneously detectable only by supervising the relative fluorescence of these 2 color.

[0062]

Example 10 The catabolic rate and the guidance scale factor of dEGFP By mutating the key amino acid of the PEST array of dEGFP, the variant of a large number with a different half-life was created. They were displayed as d1EGFP and d4EGFP reflecting those half-lives (drawing 9). A d1EGFP variant has the half-life of less than 1 hour, and a d4EGFP variant has the half-life of about 4 hours (drawing 10). Since EGFP-MODC 422-461 had the half-life of about 2 hours, it was displayed as d2EGFP.

[0063]

The property of the dEGFP protein which stood high as compared with EGFP is that there is more little accumulation of destabilization protein, though they show prompt metabolic turnover, consequently it is the activity of the original level. Therefore, as proved by the TNF agency NFkB activity system, d2EGFP is the imprint reporter of high sensitivity farther than EGFP. Since d1EGFP has a half-life shorter than d2EGFP, d1EGFP is high sensitivity from d2EGFP, when it uses as an imprint reporter. In order to explain this, d1EGFP and the d2EGFP gene were combined with the cAMP controlling element (CRE), and those responses to forskolin were examined about each fusion construction object. Although 4 times as many guidance as this was obtained when d2EGFP was used as a reporter, when d1EGFP was used, guidance higher than 12 times was attained (drawing 11). These results show that the high sensitivity which guided and improved is attained, so that the turnover rate of a GFP molecule is high.

[0064]

The patent or publication stated to this specification shows the level of the technical field relevant to this invention, and uses it for as reference to the same extent with having applied each publication separately to.

[0065]

this invention carries out the purpose accompanying the above and them, and it will be easily understood by this contractor that it is suitable to attain those targets and advantages. It is not for a method, operation, processing, a molecule, and a concrete compound being the examples of representation of a mode desirable at present, and

limiting the range of this invention to these examples indicated on these specifications, and a row. those change and other uses that are included by the pneuma of this invention defined by the claim -- this contractor -- obvious -- it will be .

[Brief Description of the Drawings]

[Drawing 1]

It is the typical map of EGFP and EGFP-MODC fusion protein. EGFP is united with the C terminus field (the amino acid of 376-461, 376-456, or 422-461 is included) of MODC. It is CHO about these fusion protein. K1 In a Tet-off cell, it was made discovered, and those fluorescence intensity was measured under the fluorescence microscope.

[Drawing 2]

The fluorescent stability of EGFP inspected with the fluorescence microscope under existence of a cycloheximide in the cell and EGFP-MODC 422-461 is shown. It is CHO at the vector which discovers these 2 protein. K1 The transfection of the Tet-off cell was carried out. the cell which carried out the transfection 24 hours after -- a 100mg [/ml] cycloheximide -- 0, 1, 2, and 3 -- and it processed for 4 hours

[Drawing 3]

Fluorescent stability analysis of EGFP by the flow cytometry and EGFP-MODC 422-461 is shown. CHO K1 The transfection of the Tet-off cell was carried out by EGFP and EGFP-MODC 422-461. the cell which carried out the transfection 24 hours after -- a 100microg [/ml] cycloheximide -- 0, 1, and 2 -- and it processed for 3 hours The processed cell was collected by EDTA and 10,000 cells were used for FACS analysis.

[Drawing 4]

It is the graph which gathered the flow-cytometry data from drawing 3. An EGFP cell proves maintaining fluorescence to the cell which carried out the EGFP-MODC422-461 transfection of this losing fluorescence promptly after cycloheximide processing.

[Drawing 5]

It is the photograph of the Western blot analysis of the protein stability of EGFP and EGFP-MODC 422-461. The cell collected in the flow cytometry was used for manufacture of a lysis object. SDS gel electrophoresis of the lysis object is carried out, and it was moved to the film. The monoclonal antibody to GFP detected EGFP and EGFP fusion protein.

[Drawing 6]

It is the typical map of the PEST array of fusion EGFP-MODC 422-461 having shown the position of variation.

[Drawing 7]

CHO which discovers EGFP, EGFP-MODC 422-461, and a PEST variant and which carried out the transfection K1 In a Tet-off cell, it is the table which summarized the result which measured and obtained duration of a fluorescence signal. The transfection was performed by the method mentioned as the example 2 in the CHO/tTA cell. 24 hours after and a cell -- a cycloheximide -- 0 and 2 -- and it processed for 4 hours and the FACS caliber (Caliber) analyzed fluorescence

[Drawing 8]

The ** type view of d2EGFP, dECFP, and dEYFP is shown.

[Drawing 9]

Construction of a destabilization EGFP variant is shown.

[Drawing 10]

The fluorescent stability of EGFP and a dEGFP variant is shown.

[Drawing 11]

The guidance increase by CRE-d1EGFP and CRE-d2EGFP is shown.

[Translation done.]

CLAIMS

[Claim(s)]
[Claim 1] Fusion protein which is fusion protein containing fluorescence protein and has the half-life which does not exceed about 10 hours.
[Claim 2] Fusion protein according to claim 1 chosen from the group which fluorescence protein becomes from EGFP, ECFP, and EYFP.
[Claim 3] Fusion protein according to claim 2 with which fusion protein contains the PEST array content portion of the C terminus of the rat ornithine decarboxylase (MODC) united with fluorescence protein.
[Claim 4] Fusion protein containing the amino acid chosen from the group which the PEST array content portion of the C terminus of the rat ornithine decarboxylase becomes from MODC 376-461, MODC 376-456, MODC 422-461, P426 A/P427A, P438A, E428 A/E430 A/E431A, E444A, S440A, S445A, T436A, D433A/D434A, and D448A according to claim 3.
[Claim 5] protein -- array number: -- the fusion protein according to claim 3 which has the array shown in 1
[Claim 6] The isolation DNA molecule which carries out the code of the fusion protein according to claim 1.
[Claim 7] DNA which carries out the code of the fusion protein which is DNA according to claim 6 and is what is chosen from the group which fluorescence protein becomes from EGFP, ECFP, and EYFP.
[Claim 8] DNA which carries out the code of the fusion protein containing the PEST array content portion of the C terminus of the rat ornithine decarboxylase (MODC) which is DNA according to claim 7 and was united with fluorescence protein.
[Claim 9] DNA according to claim 8 chosen from the group which the PEST array content portion of the C terminus of the rat ornithine decarboxylase becomes from MODC 376-461, MODC 376-456, MODC 422-461, P426 A/P427A, P438A, E428 A/E430 A/E431A, E444A, S440A, S445A, T436A, D433A/D434A, and D448A.
[Claim 10] Array number: Isolation DNA according to claim 8 which has the array shown in 2.
[Claim 11] The vector which may discover a isolation DNA molecule according to claim 6.
[Claim 12] The vector according to claim 11 in which a vector contains an inductive promotor.
[Claim 13] The vector which may discover a isolation DNA molecule according to claim 10.
[Claim 14] The vector according to claim 13 in which a vector contains an inductive promotor.
[Claim 15] The vector according to claim 14 whose promotor is tetracycline inductivity.
[Claim 16] How to be the manufacture method of the stable cell lineage which discovers fluorescence protein, and include the process which carries out the transfection of the cell by the vector according to claim 11.
[Claim 17] The stable cell lineage manufactured by the method according to claim 16.
[Claim 18] the method of carrying out the assay of activation or deactivation of an imprint or a translation element with transient fluorescence reporter protein -- it is -- the expression vector containing the fluorescence protein fusion protein which has the half-life which does not exceed about 10 hours -- a cell -- a transfection -- carrying out -- that time -- fusion protein -- the bottom of those promotors, an imprint, or the influence of a translation element -- it is --; -- and -- A method including the process which detects existence of the fluorescence in those cells,
[Claim 19] The way according to claim 18 the amount of the fluorescence which exists in a cell is the scale of the fluorescence protein to discover.
[Claim 20] It is the method of carrying out the assay of activation or deactivation of a promotor, other imprints, or a translation element with transient fluorescence protein reporter protein. The transfection of the cell is carried out by the expression vector containing the fluorescence nature fusion protein which has the half-life which does not exceed about 10 hours, fluorescence nature fusion protein is under those promotors, an imprint, or the influence of a translation element in that case, and it is;.
the cell which carried out the transfection -- the purpose compound -- processing --; -- and -- Method including the process which carries out the assay of the influence detects change of the fluorescence at the time of processing a cell with the purpose compound, and the purpose compound affects activation or deactivation of those imprints or a translation element by this.

[Claim 21] It is the method of investigating a cell lineage. The transfection of the undifferentiated cell is carried out by the vector which discovers the destabilized fusion protein according to claim 1, and it is;.
an undifferentiated cell increases these undifferentiated cells under the conditions which become a differentiated cell -- making --; -- and -- Method including the process which detects un-existing, the existence, or the position of the fluorescence in a differentiated cell.
[Claim 22] It is the method of using fusion protein according to claim 1 for a cell localization examination. The transfection of the cell was carried out by the expression vector containing the GFP fusion protein which has the half-life which does not exceed about 10 hours, fusion protein is combined with the cell localization element by presumption in that case, and it is;.
these cells are increased -- making --; -- and -- Method including the process which detects the position of the fluorescence in a cell.

[Translation done.]